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Prereplicative Complexes Assembled *In Vitro* Support Origin-Dependent and -Independent DNA Replication

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 09 December 2013

Thank you for submitting your manuscript on in vitro DNA replication with purified pre-RCs for our editorial consideration. We have now received the comments of three expert referees, which you will find copied below for your information. As you will see, the referees acknowledge the importance of this technical achievement as well as the potential interest of some of the follow-up findings obtained with this system. Nevertheless, they also raise a number of substantive concerns that would need to be satisfactorily addressed before publication in The EMBO Journal would be warranted. Since these issues appear to be in principle straightforward to address, I would like to invite you to prepare a revised version of the manuscript in response to the referees' comments and criticisms. Please keep in mind that it is our policy to allow only a single round of major revision and that it is therefore important to carefully respond to all points at this stage. Before resubmitting, please also make sure to carefully edit and proofread the manuscript to correct the various formatting and referencing errors noted by the reviewers. Furthermore, when preparing your letter of response to the referees' comments, please also remember that this will form part of the Review Process File, and will therefore be available online to the community.

We generally allow three months as standard revision time, and it is our policy that competing manuscripts published during this period will have no negative impact on our final assessment of your revised study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

Thank you for the opportunity to consider this work, and please do not hesitate to contact me in case you should have any additional question regarding this decision or the reports. I look forward to your revision.

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REFEREE REPORTS:

Referee #1:

"Prereplicative complexes assembled in vitro support origin-dependent and -independent DNA replication" by On et al.

The initiation of chromosomal DNA replication in eukaryotic cells consists of consecutive two steps; formation of the pre-replicative complex (pre-RC) in the absence of cyclin-dependent protein kinase (CDK) and assembly of replication proteins on origins in the presence of CDK and Dbf4-dependent protein kinase (DDK). Recent development of in vitro replication system using budding yeast extract and reconstitution of the pre-RC from its purified components devised the way to investigate the initiation step of eukaryotic DNA replication in detail. However, it had been unknown whether the reconstituted pre-RC is functional for initiation of DNA replication. In this manuscript the authors showed that the reconstituted pre-RC is functional in in vitro replication system. Moreover, they find that origin specificity is low if they used high concentration of ORC in the absence of competitors although the binding of ORC to DNA alone defines the replication origins. In addition, the authors analyzed the product quite extensively and found that DNA molecule is fully replicated in the bead-free system. The experiments were carried out carefully and well documented. The results shown in the manuscript support their conclusion.
This manuscript enable us considering the knowledge of the reconstituted pre-RC reported here and before as a biologically functional pre-RC and also provides the tool to improve the in vitro replication system.
Taken altogether, this manuscript advances our knowledge of eukaryotic DNA replication significantly and opens the way to analyze the initiation step in detail. I thus support publication of this manuscript with slight modification.

(Specific points)
1. There are some mis-quotations of Figures.
1) p.13, line 11, lanes 8-10; there are no lane 8-10 in Fig. 5A. Lanes 3-5 in the right panel of Fig. 5A should read them.
2) p.14, line 1. Fig. 5B should read Fig. 5D.
3) p.17, line 15. Fig. 7C should read Fig. 7B.
4) p.17, line 3 from the bottom. Fig. 7D should read Fig. 7C.
2. p.19, the last line. Masumoto et al. MCB 20, 2809 (2000) should be quoted for non-movement of Dpb11.
3. p.12, line 11. Plasmid DNA relaxed by topoisomerase I does not migrate with supercoiled molecule in the presence of EtBr. Actually lanes of Fig. 4C showed that these molecules migrate slight differently.
4. Introduction, "pre-RCs need to be removed from the first extract which contain inhibitors of origin firing including the CDK inhibitor Sic1". Is there any evidence for this description?
5. Although this is not mandatory, readers will appreciate some more information of Mass-spec results. For example, Fig.6. A-C, what is the plot between DPB11 and SLD2? How about Pol epsilon in Fig.6D?

Referee #2:

The manuscript by Diffley and colleagues describes an in vitro replication assay that utilizes the author's previously described method for assembling Pre-RCs on DNA using purified proteins rather than a G1 extract. Their findings show that the resulting replication is semi-conservative, relies on DDK phosphorylation, polymerase activity and active CDK, all hallmarks of replication in eukaryotes. Because they use purified proteins to assemble Pre-RCs, the authors avoid inhibitors present in G1 extracts used in a previous study and are able to replicate unattached plasmids by the addition of DDK and S phase extract directly to the pre-RC associated DNAs. In these conditions, they observe a majority of full-length products, which was not observed in the previous extract based system. Although they do not observe origin specificity, this is not without precedent in their Pre-RC system and manipulation of ORC and competitor DNA levels allow some level of

specificity to be obtained. This method represents the next step towards a full reconstitution of DNA replication.

The authors also utilize mass spectrometry to analyze the composition of proteins associated with the DNA after S phase extract addition with or without intervening DDK addition. These data identify factors that are enriched upon replication initiation. The authors focus on factors that are enriched in the +DDK over the -DDK reaction (i.e. above the diagonal) as these are likely to be involved in DNA replication. The authors should discuss why some of the "firing-factors" are present in both conditions but only modestly enriched when DDK is present, while others are exclusively present in the +DDK conditions. For example, two factors that they overexpress, Dpb11 and Sld3, have similar iBAQ values in the +DDK condition, but Dpb11 is only observed in the +DDK sample, while Sld3 is observed in both the +DDK and -DDK samples (albeit more so in the +DDK sample). The authors should do a better job of discussing the implications of the different DDK-dependence of the replication factors identified in the mass spec experiments.

In connection with their mass spec data, the authors claim in the discussion that "virtually all of the other identified components of the RPC" were found. This suggests that some were not. If so, the authors should indicate and comment on what was missing. The authors also state that "Surprisingly few proteins other than known replisome components and firing factors were found associated in a DDK-dependent manner." Given that only a subset of the proteins that are DDK-dependent are labeled and there is no table identifying all of the proteins identified it is hard to evaluate this statement. The authors should should include a table of all proteins found or remove this statement.

The final figure in the paper discusses the origin dependence of the assay. Although it demonstrates that the replication system can be made more origin specific in the presence of competitor DNA and decreased ORC addition to the helicase loading reaction, the authors claim that this "replication competence correlates completely with ORC binding and pre-RC assembly" and that "origin-specificity appears to be determined at the pre-RC assembly step." As they do not monitor ORC binding or pre-RC assembly in this study, this conclusion cannot be supported. The effect of competitor DNA on specificity could be at multiple stages of the reaction. Based on the data shown it remains possible that there are events downstream of pre-RC formation that are being influenced by origin sequences (as is proposed in the co-submitted paper by Remus and colleagues). The authors need to expand the experiments in the manuscript to support these statement.

Other points:

1. Many figures in the text are mislabeled. In the text, a reference is made to Fig 5A lanes 8-10. Such lanes do not exist. There is also a reference to Fig 5D that does not exist. The mass spec data is contained in Fig 6, but is referred to as Fig 5. Figure 7 figures referred to in the text do not correspond to the proper experiments. There are probably other mistakes as well.

2. The authors discuss DDK phosphorylation efficiency, but two methods for purifying DDK are described and which type of DDK is used in these experiments should be indicated. For example it is possible that the different preparations are more or less active. The authors should clarify this, or state that both preparations had equal efficiencies and the proteins were used interchangeably.

3. In Figure 1C, the phosphorylation of Mcm6 appears to be incomplete (unlike Mcm4). Since they authors suggest that all pre-RCs are converted into pre-ICs (which requires DDK phosphorylation of Mcm2-7). Do the authors think that phosphorylation of only Mcm4 is sufficient for pre-IC formation or is it possible that once S phase extract is added additional phosphorylation occurs?

4. In Figure 4, the -EtBr and +EtBr labels should be moved because it appears that they refer to parts A and B, rather than C.

5. Because the authors treat the Pre-RCs with DDK alone in Fig 1, they should make it clearer that in the Mass spec experiment, they are in the presence of S phase extract with or without DDK rather than just Pre-RCs with or without DDK.

Referee #3:

Eukaryotic DNA replication initiates from multiple origins of replication. In buddying yeast, origins are well-defined by specific DNA sequences which support origin recognition complex (ORC) binding and loading of the inactive core of replicative helicase (Mcm2-7). During replication initiation this inactive helicase is remodelled and activated due to action of S-phase kinases and binding of additional factors. As DNA replication is one of the most fundamental processes in life it is highly conserved through evolution and thus often studied in simpler eukaryotes or model systems. Much of what is known about this process comes from studies in buddying and fission yeast and the cell free system of Xenopus laevis egg extract. The last 5 years have seen an explosion of a new model system in the replication field - the buddying yeast cell free reconstitution system. The first step of replication - the loading of the inactive helicase onto exogenous DNA (pre-RC establishment) has been reconstituted previously with both G1 arrested cell extract and with purified proteins, and the extract loaded helicase has also been shown to be able to be activated by S-phase arrested extract.
The present manuscript is taking this system a step further. Kin and colleagues show that Mcm2-7 complexes, loaded onto DNA by the purified proteins, can also initiate and importantly lead to complete, though inefficient, semiconservative replication in S-phase arrested cell extract. Like with previously studied extract-loaded pre-RCs, reconstituted pre-RCs require proper Mcm2-7 loading, DDK phosphorylation of Mcm2-7, CDK activity for replication initiation and their processing leads to association of the expected set of replication factors. All these indicate that the double-hexamers of Mcm2-7, reconstituted on DNA with a defined set of purified proteins, are true intermediates of replication reaction initiation. Most of the characteristics of this system therefore follow known regulatory mechanisms acting within cells and in vitro with extract-assembled pre-RCs. One difference of this system is that plasmid DNA which does not contain canonical origin DNA sequences can not only load pre-RCs (as shown previously) but also activate these pre-RCs for replication.
Altogether, this manuscript presents a thorough characterisation of a newer version of the cell-free buddying yeast reconstitution system, which is now able to establish complete plasmid replication. It does not yet however provide new exciting insights into the process of DNA replication.

Major points:
1. In introduction authors state: "Xenopus laevis egg extracts can replicate exogenous DNA (Blow & Laskey, 1986; Walter et al, 1998) and this system has been important for understanding replication mechanism and control. However, fractionation of these extracts has not been possible." Most of the early work in X.l. egg extract to characterize DNA replication has been done through biochemical fractionation of egg extract and so this statement is wrong.
2. p. 6. - Authors show that DDK phosphorylation of Mcm2-7 does not lead to double-hexamer separation (Figure 1). The same observation has been previously published in X.l. egg extract (Gambus et.al 2011), where DDK phosphorylated Mcm2-7 complexes on chromatin still form double-hexamers. Authors should acknowledge and discuss this previous finding.
3. The authors suggest that the inefficiency of replication in their system likely results from limiting amounts of Mcm10 protein. This can be easily tested by overexpression of Mcm10 or by supplementing extract with recombinant Mcm10.
4. The DNA replication in the cell free extract base system described in Heller et al 2011 (which is a base for the system reported here) depended on the presence of origin sequences (ACS) within the replicated plasmid, but they used a different plasmid (pUC19ARS1). It would be informative to test whether this particular plasmid can also replicate without the need of replication sequences in the system derived here. It would allow the authors to distinguish whether these two cell-free systems differ in this regulatory manner or not.
5. In order to draw any conclusions about RPA enrichment upon aphidicolin treatment conditions (Fig 6) authors should provide a positive control - conditions able to increase RPA DNA binding. As they point out themselves most of the RPA binding may come from background, non-specific beads binding. Moreover, as this experiment has been performed with plasmids bound to beads, the distance of DNA for the helicase run-off may be restricted by attachment of DNA to the beads.
6. Supplementary Table 1 contains a list of yeast strains used in this study and not "the relative abundance of proteins recruited to pre-RCs under a variety of conditions (Supp. Table 1)" as stated on p. 14.

Minor points:
1. p. 6 - "Fig. 1C shows that Mcm4 and 6 in the double hexamer could be extensively

phosphorylated with this kinase, and these conditions were used in all subsequent experiments". Which conditions? - the figure shows testing of a number of DDK conditions.
2. Figure 1 a/b is a recapitulation of previously published data and could be put into the supplementary data.
3. Could authors comment why they observe much more enrichment of Dpb11, Sld2, Sld5 and Sld7 on pre-RCs DNA than Cdc45 and Sld3? (Figure 6).
4. Figure legend in Figure 7 has point C described twice and messed up letters further on.
5. Figure 7 - are the observed effects reproducible? What are the error bars on the quantified replication values?
6. There is no authors contribution section, no conflict of interest statement and no references under the "reference" heading in supplementary materials and methods.

1st Revision 17 December 2013

Response to referees

Referee 1:

We were pleased this referee felt our paper 'advances our knowledge of eukaryotic DNA replication significantly' and supported publication of our paper 'with slight modification'.
1. We have fixed all the mislabelled figures.
2. We have included the Masumoto et al reference.
3. We have gone back to the original data and remade the figure. The supercoiled, labelled DNA can now be seen to co-migrate with the supercoiled DNA. The referee is correct that the relaxed and supercoiled DNA do migrate slightly differently (Fig. 4C, lanes 7,8). These molecules have very different linking numbers and it is possible that the amount of ethidium bromide isn't saturating. Nonetheless, this figure clearly makes thepoint that a large fraction of our replicated DNA is covalently closed.

4. The referee is correct. The sentence has been reworded to remove the overstatement as follows: '*Attachment of the DNA to beads allows removal of pre-RCs from the G1 extract, which contains inhibitors of origin firing including the CDK inhibitor Sic1, before transferring to the S phase extract. In this paper we show that pre-RCs assembled on plasmid DNA with purified proteins can effectively replace the G1 extract.*'

5. We have now included the mass spec data in an Excel file so interested parties can sort and examine the data. The protein between Dpb11 and Sld2 is Msc6. It's annotated as being involved in meiotic recombination and localises to mitochondria. It is non-essential and doesn't have any documented interactions with known replication proteins, so we didn't highlight it. There are a few other proteins like this above the diagonal, but very few. DNA pol epsilon is on the diagonal in Fig. 6D, so we didn't highlight it.

Referee 2:
This referee indicated that our work represents 'the next step towards a full reconstitution of DNA replication'. Two of this referees main concerns involved our mass spec data. Firstly, this referee wanted a discussion of why 'some of the firing factors are present in both conditions but only modestly enriched when DDK is present'. It is true that many of the firing factors (e.g. Dpb11, Sld2, Sld5) look as though they are absent from the –DDK sample (i.e. are along a line at the left side of the graph), whilst others (e.g. Dpb2, Pol2, Sld3, Cdc45, DDK) look like they are already present in the absence of DDK but are enriched +DDK. However, there are two reasons for why we don't believe there is a significant difference between these groups:

1) The group that looks only 'modestly enriched' are actually all very highly enriched. The y-axis is log10 transformed. Consequently, every unit represents a 10-fold difference in abundance. The least enriched of the group, Sld3, is 1.8 units higher in the +DDK sample, which represents a 63-fold enrichment. There may be reasons why there is a small amount of background binding without DDK for this group; pol epsilon may bind to DNA even without DDK, for example, and background DDK may represent the inactive DDK in the extract, which may be recruited even though it is inactive.

Nonetheless the enrichment of this group is dramatic. To clarify this, we have added two new sentences. On p. 14: '*Because the iBAQ values are log10 transformed, each unit on the y-axis represents a 10-fold difference in abundance.*' And on p. 15: '*Although it appears some firing factors (Dpb2, Pol2, DDK, Cdc45 and Sld3) are also present without DDK, because the yaxis is log10 transformed, each is enriched by at least 60-fold in the presence of DDK.*' Hopefully this clarifies the data.

2) Proteins in the group that looks 'exclusively present in the +DDK conditions' actually all have iBAQ scores of 3 (note that the x-axis starts just below 3, not zero). The reason for this is that all proteins with iBAQ scores <3 were assigned a value of 3, in part to simplify the graphs. We have added the following in the figure legend to make this clear: '*Note that the y-axis is log10 transformed. Thus, each unit represents a 10-fold difference in abundance. Proteins with iBAQ values <3 were all assigned a value of 3 to simplify the graph.'*
This referee also requested a table containing the mass spec data. We have now included an Excel file containing all the mass spec data.
The final major point this referee made was that, because we hadn't monitored ORC binding/pre-RC assembly in Figure 7, we shouldn't make the claim that 'origin specificity appears to be determined at the pre-RC assembly step'. We agree that this was an overstatement. We have removed this clause from the sentence and we have re-written the paragraph in the Discussion on this subject to more accurately reflect our data:
'Our results contribute to this idea: complex functional DNA replication origins are not required for DNA replication of naked DNA in our extract system. Instead, replication competence appears to correlate with ORC binding since conditions which promote sequence specific ORC binding (i.e. reduced ORC concentrations and the presence of competitor DNA) also promote origin dependence…
Moreover, we cannot rule out the possibility that some simple DNA sequences may contribute to events downstream of pre-RC assembly, such as origin melting.'

Other points:
1. We have fixed all of the mislabelled figures.
2. We have included the following sentence on p 6: 'DDKs from both sources were similarly efficient and were used interchangeably.'
3. Although Mcm6 appears as 2 bands after DDK treatment, both are DDK phosphorylated (i.e. migrate slower than unphosphorylated Mcm6) thus, we cannot draw any conclusions about the roles of Mcm4 and 6 phosphorylation.
4. We have moved these labels to the bottom of the figure.
5. We have added the following sentence to the Figure Legend: '*In all cases, pre-RCs were incubated with S phase extract as described in Experimental Procedures.*' To make this point clear.

Referee 3
This referee raised 6 Major points.

1. The referee is correct; this was a misstatement. It was meant to convey that the Xenopus system had not been fully deconstructed to individual components. I have re-written the sentence as follows: '*However, reconstitution of replication with purified proteins has thus far not been accomplished*.' which I believe is correct.

2. Though it is true Gambus et al found that DDK treatment did not convert the double hexamer into a smaller form (i.e. single hexamer), they did not unambiguously show the MCM remained as a double hexamer, rather than being converted into single hexamers with associated proteins which fractionate on gel filtration with a molecular weight similar to the double hexamer. We have re-written this sentence to take this into account as follows: '*This is consistent with work from Gambus et al. who showed that chromatin-bound Mcm2-7 complexes, which appeared by gel filtration to be double hexamers, were not converted to a smaller form after DDK treatment in a Xenopus egg extract (Gambus et al, 2011).*'

3. We have tried adding recombinant Mcm10 to extracts, which did not improve replication efficiency. This is a negative result which might indicate that our recombinant Mcm10 is inactive, that Mcm10 isn't the only limiting factor in the extract or that Mcm10 isn't a limiting factor in extracts. We removed the word 'likely' to describe Mcm10 as a candidate for a limiting factor, but

otherwise didn't change the text, because this was a discussion point, not a conclusion from our data, and we still feel it is worth making this point.

4. We do not conclude that replication is entirely non-specific in our system. Indeed, we specifically discuss the possibility that there may be some simple sequences in bacterial plasmids and lambda DNA that may promote replication. However, these sequences do not function as replication origins in yeast; consequently, our system has relaxed sequence requirements relative to the *in vivo* situation. The plasmids we used are very similar to the plasmid used by Heller et al., with a backbone of pBluescript rather than pUC19. A bigger difference between the two systems is that only ~1/3 of the plasmid molecule appears to be accessible to the replication machinery in the Heller et al system, because of the DNA bead attachment.

5. We did point out in the original version that RPA binding showed little or no DDK dependence and was likely, therefore, to be mostly background. Nonetheless, we have re-written this paragraph to further emphasise that we cannot draw any significant conclusion about the coupling of helicase activity and DNA synthesis:
'*None of the RPA subunits are enriched under these conditions. This may suggest that significant uncoupling of unwinding and polymerisation does not occur in these extracts, in contrast to the situation in Xenopus egg extracts (Byun et al, 2005; Pacek & Walter, 2004). However, there was quite a high background of RPA binding even in the absence of DDK (Fig. 6C) suggesting that most RPA binding is non-specific in these extracts. Thus, further work is required to determine whether or not unwinding and DNA synthesis are tightly coupled in this system.*'

6. Apologies. An Excel table with all mass spec results is now included.

Minor points:
1. This sentence has been re-written as follows: '*Fig. 1C shows that Mcm4 and 6 in the double hexamer could be extensively phosphorylated with this kinase, and the maximum amount of kinase (100nM) in this experiment was used in all subsequent experiments.*'
2. The replication on beads takes up very little space in the figure and is a useful place to start our narrative. We would rather not put it into Supplementary material.
3. All of the pre-IC proteins, including Sld3 and Cdc45 are present at very similar levels +DDK (iBAQ around 9.5-10) and all are enriched at least 60-fold – see our response to referee 2 for further explanation.
4. Apologies. This has been fixed.
5. These effects are entirely reproducible. There are no error bars because this represents quantification of the single experiments shown in (D) and (F).
6. These have all been fixed.

Acceptance letter 10 January 2014

Thank you for submitting your revised manuscript for our consideration. It has now been seen once more by two of the original referees (see comments below), and I am happy to inform you that there are no further objections towards publication in The EMBO Journal.

Referee #2
The revised manuscript is appropriate for publication.

Referee #3

I looked through the authors response letter and the corrected version of the manuscript and I am of an opinion that the authors satisfactorily answered the initially raised specific criticisms.

